Microfluidic based biosensing for *Escherichia coli* detection by embedding antimicrobial peptide-labeled beads

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A B S T R A C T

Due to their ability of effective binding to multiple target microbes, the antimicrobial peptides (AMPs) have recently received lots of attention as an alternative to antibodies for detecting bacteria. We developed a new biosensing method to detect *Escherichia coli* (*E. coli*) by implementing a microfluidic chip designed with a weir inside the channel, in which AMP-labeled microbeads were embedded. We characterized the detection rate of the stained *E. coli* within a certain period of time to examine the detection effectiveness of our device. As the flow rate of the bacterial suspension increases, the detection time to reach the saturation level decreases to less than 30 min, suggesting rapid detection, while the detection efficiency is maintained at a similar level. Except with very low concentrations of *E. coli* (<10\(^3\) cells/ml), both the detection time and the efficiency do not depend on the *E. coli* concentration. Our method has the potential to be developed as a novel biosensing platform for rapid and accurate detection of pathogens.© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Pathogen detection serves as a fundamental tool in many fields, including clinical diagnostics, pathology, drug discovery, disease outbreaks, food safety, and water monitoring. Microfluidic based pathogen detection platforms offer advantageous detection in view of their miniaturization, small sample volume, portability, rapidity, and point-of-care diagnosis [1]. Antibodies are widely utilized for the detection, identification, and quantification of pathogens [2–5]. However, it is often necessary to use a lot of animals for the production of antibodies, and these antibodies have several limitations in their chemical or physical stability, usefulness for all potential analytes, quality-assured preparations, and effective cost. As the emerging alternatives to antibodies, one can consider antimicrobial peptides (AMPs) [6–8], aptamers [9,10], real-time quantitative polymerase chain reaction (PCR) [11], primers with micro-PCR chip [12], and peptide nucleic acid (PNA) probes [13].

Among these, the natural AMPs produced by plants, insects, mammals, and microorganisms serve several attractive advantages, including a broad spectrum of antimicrobial activity, increased bacterial resistance, and reaction with a very low concentration. The AMP-binding activity is related to the cationic and amphiphilic nature of AMPs. A total positive charge accumulates at polyanionic bacterial cell surfaces that contain acidic polymers, such as lipopolysaccharide (LPS) and wall-connected teichoic acids in Gram-negative and Gram-positive bacteria, respectively. Subsequently, the AMPs contact the anionic surface of the cytoplasmic membrane and insert in a way of getting on the interface of the hydrophilic head groups and the fatty acyl chains of membrane phospholipids [14]. However, the influence of the membrane lipid composition on the specificity of the AMPs toward a given organism is not yet understood. In addition, the action of many peptides cannot be explained by disruption of membrane permeability barriers, as discussed in some reviews [14,15]. Although the mechanism of action for the AMPs and resistance is lack of understanding, AMPs have been used as a good biosensing tool to detect a variety of microorganisms.

Since the AMP magainin II was discovered from the skin of the African clawed frog *Xenopus laevis* [16], most studies of AMPs have been focused on magainin II. However, a later work applied the AMP magainin I as a recognition element for bacteria, postulating that an array consisting of multiple AMPs would potentially be able to detect a higher number of target species than an array with a corresponding number of antibodies [6]. As compared with other AMPs, magainin I demonstrated stronger antimicrobial activity through the disruption of the microbes’ membranes after it bound to
components of the membranes [7]. Recently, Mannoor et al. [8] first used AMP for the detection of pathogenic bacteria in microfluidic flow, using impedance measurement as a label-free and portable biosensor platform. In there, the AMP magainin I was immobilized on arrays of gold electrodes for the detection of pathogenic bacteria. Moreover, the stronger antimicrobial activity, as compared to other AMPs, permits magainin I to be used as an AMP-coated polymer brush [17] and an anti-biofilm [18].

Microbead-based microfluidic devices have been widely used in the field of bioassay, because they have advantages in disposability, specificity, and rapid detection. Microbeads of agarose [19,20], glass [4], magnetic materials [5,21,22], silica [23], and polystyrene [24] have normally been used as the either solid or mobile support in microfluidic devices to trace biomolecules. Moreover, for effective packing microbeads, the microchannel needs a pillar or a weir structure [25,26]. Compared to the geometry of flat plates, the microbeads in microfluidic devices with AMP-binding activity can be applied as a novel sensitive method of bacteria detection, due to the increase in the available surface area for binding to microorganisms.

In this study, we have developed a new method for the immobilization of AMPs on microbeads to detect bacteria by combining microfluidic techniques. Embedding the AMP-labeled beads into a microchannel designed with a weir provides the sensitive and accurate pathogen detection. Here, nonpathogenic *Escherichia coli* was selected as the model bacterium. For *E. coli* suspension, the effects of its flow rate and concentration on the detection rate were analyzed for a certain period of time, and the detection efficiency was estimated accordingly. Our device used in this work is relatively simple and can detect *E. coli* concentrations of 10^3 cells/mL (i.e., 1 bacterium/µL) within 30 min, suggesting that it has the potential to be developed for the rapid pathogen detection as well as for quantitative analysis of the AMP-bacteria interaction.

2. Material and methods

2.1. Preparation of AMP-labeled beads

The AMP magainin I (GIGKFLHSAGKFGKAFVGEIMKS), used to detect *E. coli*, was chemically synthesized to contain a cysteine residue at the C-terminus by Peptron Inc. (Daejeon, Korea) with a purity >90%. A recent study has reported that the binding affinity between magainin I and *E. coli* is much lower in N-terminal immobilization compared to that of the C-terminus because of the reduced accessibility of *E. coli* to the amine-containing residues at the N-terminus [8]. The preparation of AMP-labeled beads and the binding of *E. coli* by these beads are illustrated in Fig. 1.

We prepared primary amine-functionalized glass beads (Poly-sciences Inc., PA) with diameters of 30–38 µm by mechanical sieving. They were incubated with 1.0 mM N-[γ- maleimidobutyryloxy] succinimide ester (GMBS; Fluka, Switzerland) in absolute ethanol for 30 min at room temperature (RT), followed by three repetitions of rinsing and drying. Then, the maleimide-activated beads were incubated overnight at RT with AMP magainin I (4 mg/mL) in phosphate-buffered saline (PBS, pH 7.4) to immobilize the AMPs on the surfaces of the beads [7]. Here, the PBS was prepared with 137 mM NaCl, 2.7 mM KCl, 4.4 mM Na_2HPO_4, and 1.4 mM KH_2PO_4. The maleimide-activated beads crosslinked with a sulfhydryl group of a cysteine residue at the C-terminus of magainin I to produce direct covalent bonding between them (the immobilizing step shown in Fig. 1) [6,8,18]. The prepared AMP-labeled beads were stored at 4 °C and used within two weeks.

Next, the AMP-labeled beads were bound to the *E. coli* stained with 3 µM propidium iodide (PI: Invitrogen Inc., CA) in PBS for 45 min through the direct interaction between the AMP and the bacterial surface (the binding step shown in Fig. 1). The same method is effective to the real samples of unlabeled bacteria, where

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**Fig. 1.** Schematic representation of the immobilization of AMPs on a bead and the binding of *E. coli* to the AMP-labeled bead.
real samples can also be incubated with the PI solution for prior staining. To access bacterial cell viability, appropriate fluorescent dye should be used. According to a previous report [27], the negatively charged LPS in the outer layer of Gram-negative bacteria, such as E. coli, can readily bind to the AMP. In contrast, Gram-positive bacteria do not readily bind to the AMP due to the absence of LPS. The detected E. coli stained with PI can be visualized under a fluorescence microscope.

2.2. Microfluidic chip fabrication

By applying the standard soft lithography method, polydimethylsiloxane (PDMS) glass microfluidic chips were fabricated with a weir to form microbead-embedded channels, as shown in Fig. 2. The depth of the weir is critical for retaining the microbeads in the channel, and the depth of the main channel should be slightly higher than the diameter of microbeads to pack them into a monolayer. Channel design was performed with a computer-aided design program (AutoCAD-2009) and two photomasks for weir layer and main channel were prepared for the fabrication of a master mold on a silicon wafer.

In order to create a weir structure on the master mold, we applied photolithography twice with different negative photore sist (PRs) and aligned the two layered sets during the process. The photolithography follows as consisting of the weir layer (14 μm high, 400 μm wide, and 300 μm long) patterning by weir mask on SU-8 2015 and the main channel (42 μm high, 400 μm wide, and 24 mm long) patterning by the channel mask on SU-8 2050 (MicroChem, Newton, MA). After the post exposure bake was carefully performed, the unexposed PR was removed by dissolving with the SU-8 developer and a master mold containing negatively patterned PR remained. Here, note that the unexposed area in the mid position of the main channel becomes the weir, and the difference in the PRs heights will translate into the different depths between the weir and channel.

To produce a replica, PDMS (Sylgard 184, Dow Corning, MI) mixed with the curing agent in a volume ratio of 10:1 was poured on the master mold, and then was cured against the master at 80 °C for at least 1 h. The peeled PDMS replica was punched to generate holes for the inlet and outlet. It was bonded to slide glass (Marienfeld, Germany) using an oxygen plasma generator (CUTE-1MP, FemtoScience, Korea), which was baked at 80 °C for at least 40 min and stored at RT. Finally, Teflon tubing (ID: 0.8 mm, OD: 1.5 mm) was adhered to each reservoir. The AMP-labeled beads were then injected into the microchannel using a syringe pump (Pump 11 Elite-Nanomite, Harvard Apparatus, MA), and next, the packed beads were washed for 20 min with PBS at the same flow rate before the injection of E. coli suspension. The microfluidic chip was positioned on an inverted microscope (Eclipse Ti-E, Nikon, Japan) to allow monitoring and image data acquisition of the detection of E. coli. Images were taken by a digital 3 × 14 bit color charge-coupled device (CCD) camera (AxioCam HRC, Carl Zeiss, Germany).

2.3. E. coli preparations

Nonpathogenic E. coli (ATCC 35218) was obtained from the American Type Culture Collection (ATCC). It was grown for 18 h at 37 °C in Luria Bertani (LB; Difco Laboratories, Spark, MD) broth, and diluted to the prescribed concentration. To quantify the E. coli number, we measured the optical density of E. coli culture at 600 nm (OD600) using a UV-Vis spectrophotometer (ND-2000, Thermo Scientific, DE). An OD600 reading of 1.0 corresponds to approximately 8 × 10⁸ cells/mL. In order to confirm the morphology of the cultured E. coli, PI-stained E. coli cells were observed by confocal microscope (FV-300, Olympus, Japan), as shown in Fig. 3. Typically, the cell sizes are several micrometers with a diameter of ca. 1 μm and some bacteria have long tails.

Both the number of bacteria injected (Nᵢ) and the number of bacteria discharged (Nₒ) are required to determine the detection efficiency of E. coli by our method, where Nᵢ is a known value. The collected suspension discharged from the outlet was serially diluted with PBS, and then plated onto an LB plate. After incubating LB plates for 18 h at 37 °C, we calculated Nₒ by counting the number of colony forming units (CFU) per mL and comparing them to the dilution factor. Then, Nᵢ − Nₒ corresponds to the number of E. coli detected by microfluidic chip (Nₑ), from which the detection efficiency is calculated as (Nₑ/Nᵢ) × 100 (%). All of the data were reported as mean values from three independent experiments.
3. Results and discussion

3.1. Detection of E. coli on AMP-labeled beads

We first checked the binding affinity of the AMP-labeled beads to PI-stained E. coli after incubating them for 10 min at RT. Then, the changes in the fluorescence images due to the detection of E. coli in the microchannel were observed as time proceeded, as shown in Fig. 4. During the progress of the bacteria detection, besides the specific binding between the surface of E. coli and the AMP immobilized on the surface of the glass beads, there exists also nonspecific binding caused by interspaces between beads as well as between beads and the microchannel wall. While nonspecific binding can be observed in fluorescence images from the channel embedded with unlabeled beads (Fig. 4a), those from the channel embedded with AMP-labeled beads (Fig. 4b and c) represent the total binding caused by both nonspecific and specific binding. Detecting the PI-stained E. coli ($\lambda_{ex} = 535$ nm, $\lambda_{em} = 617$ nm) was accomplished by adopting a 510-560 nm excitation filter and a 575 nm emission filter with a mercury lamp. The red color in the fluorescence images represents the detected E. coli on the surface of the beads. Detecting E. coli by specific binding clearly develops as time progresses, where the higher flow rate makes a higher degree of detection to be accomplished faster.

For accurate evaluations of the extent of specific binding (or detection), the acquired cumulative fluorescence images were inverted into black and white and the background images were subtracted using the ImageJ (National Institute of Health, MD) program. An intensity threshold was applied to estimate the fluorescent area, because of the possible variations in the intensity of the light emitted from PI-stained individual bacteria. To detect the target bacteria, approximately 170-230 of the AMP-labeled beads are packed into the channel, indicating that the cumulative fluorescence intensity should be divided by the number of packed beads.

The fluorescence intensity resulting from E. coli detection increases as time proceeds, and total detection rapidly reaches the saturation level, as shown in Fig. 5a. All data are averages from three replicate experiments, and error bars indicate their standard deviations. In comparison, Fig. 5b shows a gradual increase in the extent of nonspecific detection, which is determined by the cumulative fluorescence intensity with unlabeled beads divided by that with AMP-labeled beads. It is identified that the E. coli detection by AMP-labeled beads is advantageous for rapid detection by embedding beads inside the microfluidic chip. The nonspecific detection accounts for about 12.6-18.5% of total detection at the flow rate of 1.2 $\mu$L/min, while it increases up to about 45% at 2.0 $\mu$L/min, due to the higher convection effect on the E. coli suspension. Note that the specific detection can be considered the actual detection in our method.

3.2. Performance of E. coli detection

We examined the effects of flow rate and E. coli concentration on the performance and detection efficiency of our biosensing device. Fig. 6 shows the rate of E. coli detection by the AMP-labeled beads for flow rates of suspensions ranging from 0.05 to 4.0 $\mu$L/min. The analytical solution is available for a steady state laminar flow in microchannels having a rectangular cross-section with height and width [28]. Table 1 summarizes the hydrodynamic conditions estimated at the front of the bead packing as well as at the weir with variations of flow rates. In our experiments, Reynolds numbers are in the typical order of microfluidic system. The flow rate is inversely proportional to the residence time in the void space of the bead-packing zone, which clearly affects to the binding of E. coli to the beads.

The relationship between the cumulative fluorescence intensity and the time progress represents evidently an exponential rise with flow rates of above 1.2 $\mu$L/min. Note that detection saturates at almost 30 min after it occurs dramatically at the initial stage for high flow rates (2 and 4 $\mu$L/min). However, the binding capacity decreases remarkably at an excessive flow condition of 4 $\mu$L/min due to a deficient residence time, which is not long enough for the efficient binding between E. coli and the beads. With decreasing flow rate, the detection rate becomes slow so that it takes much longer to achieve the saturation level, indicating a linear relationship between the cumulative fluorescence intensity and the time. This feature allows us to figure out that a higher flow rate, but less than an excessive flow condition, should be applied to attain rapid detection. In comparison, other microfluidic based biosensors take more than 1 h to detect the target E. coli [6,7,11,12].

Fig. 7 shows the time-dependent curve of the fluorescence intensity for different E. coli concentrations at the injected suspension. The early saturation time of detection (cf., almost after 20 min at 10^6 cells/mL) becomes delayed with decreasing bacteria concentrations. As the bacteria concentration decreases with the same order, one can see also the attenuation tendency of decreasing in fluorescence intensity. The fluorescence intensity in the concentrations less than 10^5 cells/mL can be predicted, once we consider the saturated fluorescence intensity for each bacteria concentration that is normalized by the saturation value for 10^6 cells/mL. In Fig. 8, a change in the relative fluorescence intensity remains quite slow for the concentrations of less than 10^3 cells/mL, meaning that this
Fig. 4. The changes in fluorescence images by detecting PI-stained E. coli (10⁵ cells/mL) at times of 5, 20, and 50 min, using microchannels embedded with (a) unlabeled beads at 1.2 μL/min, (b) AMP-labeled beads at 1.2 μL/min, and (c) AMP-labeled beads at 2.0 μL/min.

Table 1
Hydrodynamic conditions applied in this study.

<table>
<thead>
<tr>
<th>Flow rate (μL/min)</th>
<th>At the front of bead packing</th>
<th>At the weir</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linear velocity (mm/s)</td>
<td>Nominal shear rate (1/s)</td>
</tr>
<tr>
<td>0.05</td>
<td>0.05</td>
<td>6.9</td>
</tr>
<tr>
<td>0.5</td>
<td>0.50</td>
<td>69</td>
</tr>
<tr>
<td>1.2</td>
<td>1.19</td>
<td>165</td>
</tr>
<tr>
<td>2.0</td>
<td>1.98</td>
<td>275</td>
</tr>
<tr>
<td>4.0</td>
<td>3.97</td>
<td>550</td>
</tr>
</tbody>
</table>

Shear rate is determined at the channel wall.
Fig. 5. (a) Real-time monitoring of E. coli (10^5 cells/mL) detection according to non-specific and total detection by unlabeled and AMP-labeled beads, respectively, and (b) changes in the extent of nonspecific detection as time proceeds. Error bars not shown in (a) are smaller than the symbol size.

level is nearly a limit of detection (LOD) for E. coli in our device. This level can be compared to the LOD for E. coli presented previously in the literature [1].

We quantified the detection efficiency of our device. Fig. 9 shows that this efficiency is ca. 87% at 10^3 cells/mL, but is maintained at a similar level of 59-64% irrespective of the applied E. coli concentrations ranging from 10^3 to 10^6 cells/mL. The detection efficiency was almost independent on the flow rate, although these data are not shown here. These results suggest that our method can be useful in dealing with bacterial cells of low concentrations close to the LOD. According to the selectivity and interbacterial strain differentiation reported in the literature [8], magainin I exhibits clear preferential binding toward the pathogenic E. coli and Salmonella, relative to the nonpathogenic E. coli and the Gram-positive bacteria, with 1.5-2 and 2 orders of magnitude difference in impedance, respectively. This selectivity is also more enhanced for the pathogenic E. coli, which shows a slightly larger response relative to Salmonella. Based on these results, the use of pathogenic E. coli may reveal a certain enhancement in the detection efficiency compared to the results displayed in Fig. 9. Moreover, our microfluidic based biosensor for detecting the whole E. coli is relatively convenient and applies the simple labeling of cells, as compared to conventional methods using antibodies or DNA/PNA probes.

Fig. 6. Time evolution of total detection of E. coli at 10^5 cells/mL with various flow rates ranging from 0.05 to 4.0 μL/min. Error bars not shown are smaller than the symbol size.

Fig. 7. Time evolution of total detection of E. coli at the flow rate of 1.2 μL/min with various concentrations of bacteria (10^3-10^6 cells/mL).

In the data presented in Fig. 7, the relative fluorescence intensity per bead is shown for various E. coli concentrations. The data shows the enhancement in detection efficiency with the higher flow rate of 4.0 μL/min. The error bars are not shown here as they are smaller than the symbol size.
4. Conclusions

We immobilized the AMP on glass microbeads to detect whole *E. coli* on the basis of the binding activity between the AMP-labeled beads and bacteria. The effective use of microbeads enables a microfluidic device to increase its detection efficiency by increasing the surface area to volume ratio for immobilization. The detection rate of nonpathogenic *E. coli* by the AMP-labeled beads embedded in the microchannel was measured by image analysis using a fluorescence microscope, with variations of the suspension flow rates and *E. coli* concentrations therein. The nonspecific detection was less than 20% of total detection at the flow rate of less than 1.2 μL/min. Its relatively slow increasing at initial time progress, as compared to the specific detection, emphasizes that the *E. coli* detection by AMP-labeled beads takes advantage of the rapid detection.

The higher flow rate provided higher binding and rapid detection by attaining a saturation level of detection within a short time of less than 30 min. However, an excessive flow rate results in a transition behavior, leading to a decrease in binding due to a very deficient residence time. Apart from the case of very low concentrations of *E. coli* (10^2 cells/mL), both the time to get saturation and the detection efficiency do not depend on the *E. coli* concentration. Our results imply that the AMP-labeled beads can be applied for the rapid and sensitive detection of low concentrations *E. coli* at an appropriate flow rate, without the use of antibodies.

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References


Biographies

Jeong Ha Yoo received his BS, MS, and PhD degrees in chemical engineering from Gachon University in South Korea, in 2004, 2006, and 2012, respectively. Since 2012, he has been working at the Laboratory of Cellular Neurobiology in the Seoul National
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Myung-Suk Chun is a principal research scientist of the KIST, leading the Complex Fluids Team at Sensor System Research Center. He received BS (1987) degree in chemical engineering from the Seoul National University, and MS (1990) and PhD (1994) degrees in chemical engineering from the KAIST. He worked as a postdoctoral associate at the University of California at Davis in 1995–1996 and visited the Max-Planck Institute at Mainz in 1999. Since joining the KIST in 1996, his areas have been including electrokinetic microfluidics, soft matters, and applications to lab-on-chips platform, closely related to either computations or experiments or both.